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Flavonoid Genes in Petunia: Addition of a Limited Number of Gene Copies May Lead to a Suppression of Gene Expression

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To evaluate the effect of increased expression of genes involved in flower pigmentation, additional dihydroflavonol-4-reductase (DFR) or chalcone synthase (CHS) genes were transferred to petunia. In most transformants, the increased expression had no measurable effect on floral pigmentation. Surprisingly, however, in up to 25% of the transformants, a reduced floral pigmentation, accompanied by a dramatic reduction of DFR or CHS gene expression, respectively, was observed. This phenomenon was obtained with both chimeric gene constructs and intact CHS genomic clones. The reduction in gene expression was independent of the promoter driving transcription of the transgene and involved both the endogenous gene and the homologous transgene. The gene-specific collapse in expression was obtained even after introduction of only a single gene copy. The similarity between the sense transformants and regulatory CHS mutants suggests that this mechanism of gene silencing may operate in naturally occurring regulatory circuits.

INTRODUCTION

Many valuable plant products, such as secondary metabolites, are synthesized via multiple enzymatic steps that usually are part of complex, branched biosynthesis pathways. To enhance the yield of a desired plant product, a channeling of precursors into specific products would be desirable. Such channeling might be obtained by blocking unwanted side branches through the expression of an antisense gene targeted against the gene(s) coding for the first enzymatic step of the side-branch pathway (reviewed in van der Krol et al., 1988b). Furthermore, product formation may be enhanced by overexpressing those genes whose products are rate limiting in the synthesis of the desired metabolites. We studied the feasibility of such product channeling in plants. As a model system, we chose the flower pigmentation pathway of petunia. Flower pigmentation provides a nice model system because changes in flower phenotype are readily scored by eye. The pigments in petunia flowers consist mainly of anthocyanins (pigmented) and flavonols (colorless) and are synthesized via the flavonoid biosynthesis pathway (see Figure 1). In petunia, this route has been characterized extensively at the genetic, biochemical, and enzymatic levels (reviewed in Schram et al., 1984; Wiering and de Vlaming, 1984). A number of genes involved have been cloned (for listing, see Mol et al., 1988). We have cloned the petunia genes for chalcone synthase (CHS; Koes et al., 1987, 1989b), chalcone flavanone isomerase (CHI; van Tunen et al., 1988), and dihydroflavonol-4-reductase (DFR; Beld et al., 1989). The expression of these genes occurs in a coordinate fashion during flower development (van Tunen et al., 1988) and precedes the accumulation of flavonoids (flavonols and anthocyanins) in floral tissue.

Until now, two approaches have been successful in manipulating flower pigmentation in petunia. First, the introduction of a maize DFR gene results in novel brick-red flowers caused by the synthesis of a pigment (pelargonidin) normally not found in petunia (Meyer et al., 1987). Second, we have shown that introduction of an antisense petunia CHS gene can inhibit flower pigmentation in both petunia and tobacco transgenic plants (see van der Krol et al., 1988a). CHS performs a key reaction in flavonoid biosynthesis by forming the basic structure of flavonoids through condensation of one molecule of 4-coumaroyl CoA with three molecules of malonyl CoA (see Figure 1). Introduction in petunia of a full-length antisense CHS cDNA gene, driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter, results in an effective reduction of CHS expression. As a consequence, a reduction in floral pigmentation is observed either evenly distributed (up to full white) or variegated in sector or ring pigmentation patterns (van der Krol et al., 1988a, 1989).

In contrast, one might be able to enhance floral colora-

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tion by overexpression of flavonoid genes. To test this, we introduced additional DFR or CHS genes into petunia. Surprisingly, we observed a reduction in floral pigmentation with a frequency of up to 25% for the DFR gene construct. The effect is obtained not only by introducing CaMV 35S-driven genes, but also, at lower frequency, by CHS genomic clones. Here we present the molecular analysis of the transgenic petunias containing extra DFR or CHS gene(s). The data indicate that the inhibition of pigmentation is caused by a specific reduction in mRNA steady-state level of the gene of which extra copies have been added to the genome. We discuss a model that may explain this sense effect and that may also relate to the mechanism of inhibition of pigmentation by antisense genes.

RESULTS

Phenotypic Effects of a Constitutively Expressed DFR Gene

To investigate the effect of an increase in DFR (see Figure 1) gene dosage and concomitant expression on floral pigmentation, a gene construct encoding a full-length DFR mRNA was introduced into *Petunia hybrida* VR. The gene was constructed by combining the constitutive CaMV 35S promoter, a cDNA copy of the petunia R27 DFR gene (Beld et al., 1989), and a nopaline synthase 3′ tail fragment (VIP178, see Figure 2) and introduced into petunia VR using standard procedures (see Methods). Of the 25 independent plants regenerated, 19 plants showed a flower phenotype indistinguishable from that of untransformed petunia VR plants. However, six plants showed a reduction in flower pigmentation in sector or ring patterns, as shown in Figure 3B. The degree of pigmentation varied among different flowers of the same plant. For instance, flowers on transformant 178-16 were either completely pigmented or had a white corolla. TLC analysis of the pigment content of the white flower tissue of some VIP178 transformants showed the presence of flavonols (data not shown). This indicates that, in these flower parts, pigment synthesis is blocked at the DFR enzymatic step (see Figure 1).

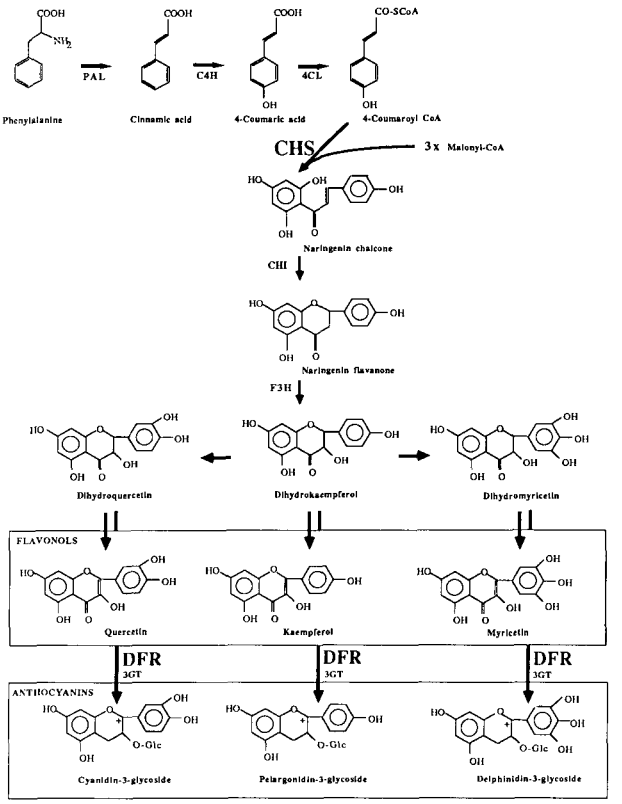

Figure 1. Schematic Representation of the Flavonoid Biosynthesis Pathway.

Enzymes are abbreviated as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol-4-reductase; 3GT, UDP-glucose:3-*O*-flavonoid glucosyltransferase. Of the compounds shown, only the tetrahydroxychalcone (also named naringenin-chalcone) and the anthocyanins are pigmented. Tetrahydroxychalcone is yellow; the color of the anthocyanins depends on substitution of the flavonoid ring structure and ranges from red (cyanidin derivatives) to orange (pelargonidin derivatives) and purple (delphinidin derivatives). Pelargonidin derivatives are not synthesized in petunia because the petunia DFR enzyme does not recognize dihydrokaempferol as a substrate.

Reduced Flower Pigmentation in DFR Sense Transformants Is Accompanied by a Specific Reduction in DFR mRNA Steady-State Level

As a first step in the elucidation of the mechanism involved in the reduction of floral pigmentation by elevating the DFR gene copy number, we measured the DFR and CHS mRNA steady-state levels in floral tissue of transformant 178-17 (pigmented flower), 178-16 (flower pigmentation varies from pigmented to white), and VR control plants. RNA was isolated from flowerbuds stage 1 to 4 and analyzed by RNA gel blotting. The blot was hybridized with ^{32}P-labeled CHS or DFR DNA (see Methods). Figure 4A shows that the DFR mRNA steady-state level in 178-17 was high compared with the VR control, and most likely was due to expression of the DFR transgene. In flowerbuds of transformant 178-16, the average DFR mRNA steady-state level was reduced compared with that of the VR control in those stages where we could not yet distinguish between flowerbuds that become pigmented or unpigmented (stage 1 to 3). Only in developmental stage 4 could we distinguish

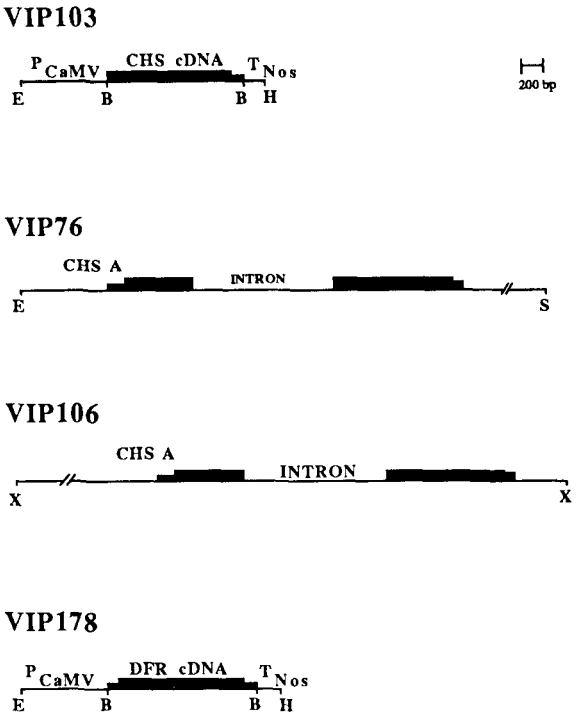

Figure 2. Structure of VIP103, VIP76, VIP106, and VIP178.

The construction of the CHS sense genes VIP103, -76, and -106 is described in Methods. In VIP103, transcription of the CHS cDNA is under control of the CaMV 35S promoter. VIP76 and VIP106 both derive from CHS-A genomic clone VIP17 (Koes et al., 1989b). VIP76 has a CHS-A-derived 5′-flanking region of 0.8 kb, whereas VIP106 has a 5′-flanking region of 4.5 kb. The construction of the DFR sense gene VIP178 is described in Methods. Transcription of the DFR cDNA is under control of the CaMV 35S promoter. For each construct the relevant restriction endonuclease sites are indicated. E, EcoRI; B, BamHI; H, Hind III; S, SalI; X, XbaI.

between pigmented and unpigmented flowerbuds. At this stage, a higher DFR mRNA steady-state level compared with VR control was found in the pigmented flower tissue, whereas, in the unpigmented flowerbuds, a severe reduction in DFR mRNA steady-state level was seen, explaining the observed block in pigment synthesis (Figure 4A; 178-16 lane 4 and 4^w). The altered steady-state level observed in 178-17 and 178-16 floral RNA was specific for the expression of DFR gene(s); Figure 4A (lower panel) shows that the CHS mRNA steady-state level was unaffected in these transformants.

In RNase protection experiments, we distinguished between the DFR mRNA derived from the DFR transgene (VIP178, transcript completely protected by the R27-DFR probe) and the petunia VR endogenous DFR mRNAs (two subfragments upon RNase protection with the R27-DFR probe). For transformants 178-16 and 178-17, we analyzed the DFR mRNA steady-state level in flowerbud stage 4 resulting from expression of the DFR transgene and that of the endogenous DFR genes. In 178-17, we measured a high expression of the DFR transgene relative to that of endogenous DFR gene(s) (Figure 4B, lane 2). In pigmented floral tissue of 178-16, we noted the same effect (Figure 4B, lane 3). In contrast, we observed a severe reduction in both the endogenous and the transgenic DFR mRNA steady-state levels in white floral tissue of transformant 178-16 (Figure 4B, lane 4). This agrees with the reduced signal for DFR mRNA found by RNA gel blot analysis (compare Figure 4A, lane 4^w with Figure 4B, lane 4). Overexposure of lane 4 (Figure 4B) shows that the residual DFR transcript derives from the DFR transgene (Figure 4B, lane 5).

DFR Leaf mRNA Steady-State Levels and Gene Copy Numbers Do not Correlate to Phenotypic Effects on Flower Pigmentation

We measured the expression of the constitutive DFR sense gene construct in leaf tissue of transformed petunia plants. Figure 5 shows the result of such an analysis. As expected, expression levels varied among different transformants, presumably due to different genome integration sites. The expression level in leaf tissue did not show an obvious correlation with flower phenotype. Both normally pigmented transformants and transformants with a clearly reduced flower pigmentation had either a high or a low DFR mRNA steady-state level in leaf tissue. (See Figure 5; there is high expression in leaf tissue of 178-8 and 178-6, whereas only 178-6 shows a reduced flower pigmentation, and there is low expression in leaf tissue in 178-12 and 178-16, whereas only 178-16 shows a reduced flower pigmentation).

For several independent VIP178 transformants, we have determined the copy number of the transgene by DNA gel blot analysis. These experiments (data not shown) did not reveal an obvious correlation between the copy number of the transgene and the phenotypic effect on flower pigmentation. For example, transformant 178-13 has approximately 10 inserts and wild-type flower pigmentation, whereas 178-16 has only one insert and a reduction in flower pigmentation (Figure 3B). Furthermore, digestion with restriction endonucleases that discriminate between internal transgene and the bordering chromosomal sequences did not reveal any detectable alterations within the transgene in the eight plants analyzed.

Phenotypic Effects of CHS Sense Genes

In a previous paper, we described the introduction and expression of a chimeric sense CHS gene (VIP103) in VR

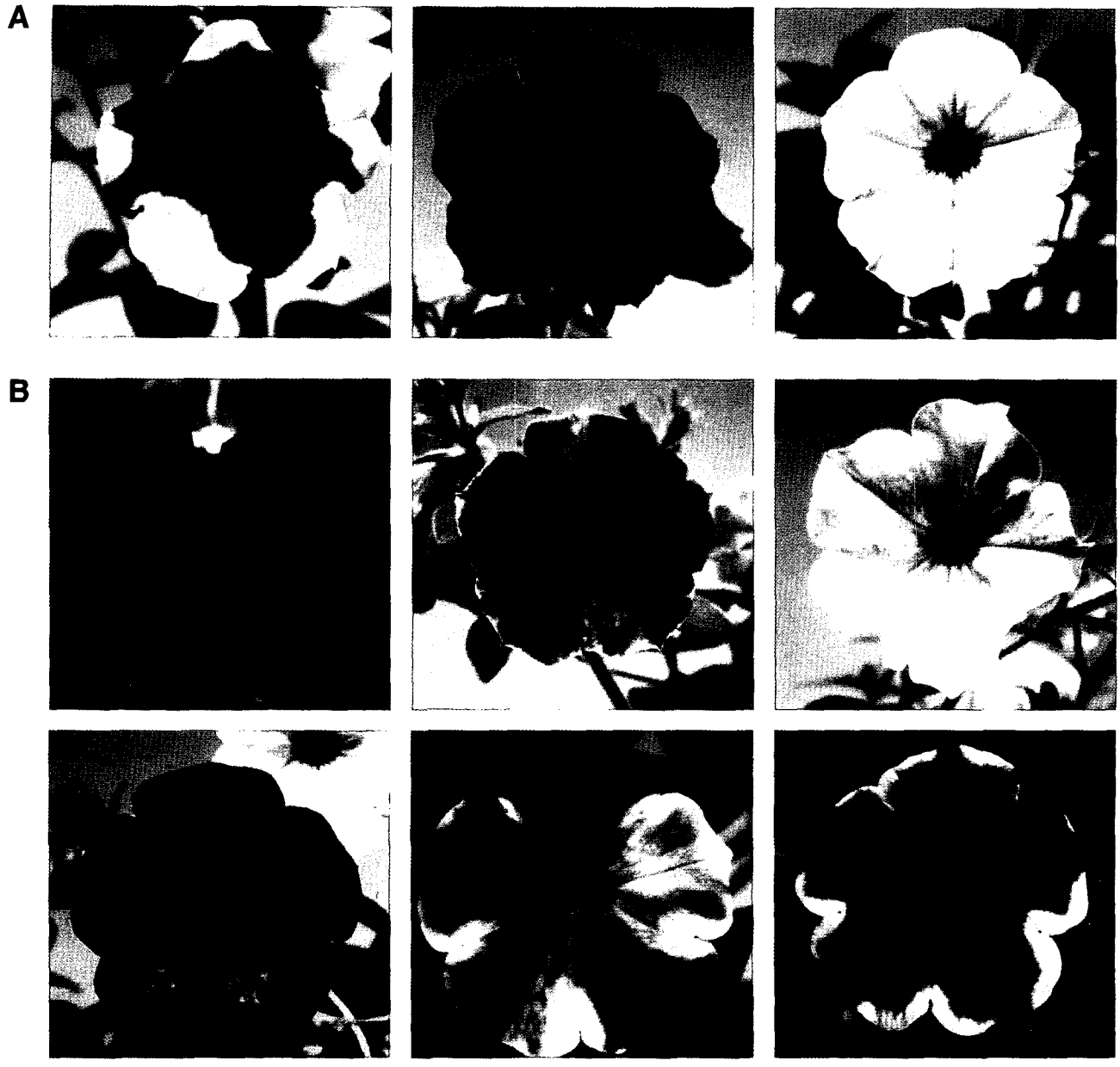

Figure 3. Flower Phenotype of the CHS Sense and DFR Sense Transformants.

(A) Shown from left to right: 103-17 (phenotype after extra illumination with 20,000 lux), 106-16, and 76-1A. Flowers on petunia VR plants transformed with the constitutive CHS gene (VIP103) initially did not exhibit an altered flower phenotype. However, when two of the VIP103 transformants were grown under extra light (20,000 lux, see Methods), flowers with white sectors developed after 2 weeks (not observed for the wild-type petunia VR). Plants containing the CHS genomic clones VIP106 (see Methods and Figure 2) showed flowers with either a wild-type pigmentation (19 plants) or an even reduction in pigment synthesis (one plant; 106-16). Wild-type flower pigmentation was found on 15 plants containing VIP76, where one plant showed white corolla tissue and reduced pigmentation of the tube (76-1A).

(B) Flowers on petunia VR plants transformed with the DFR sense gene construct VIP178 (see Methods and Figure 2) showed either an unaffected flower pigmentation (top left) or a reduction in pigment synthesis. Shown from top left to bottom right: 178-1, 178-14, 178-6, 178-16, 178-10, and 178-15. On transformant 178-16, flower pigmentation varies from fully pigmented to white. Transformant 178-15 shows an ectopic expression pattern, resulting in a white ring at the edge of corolla tissue.

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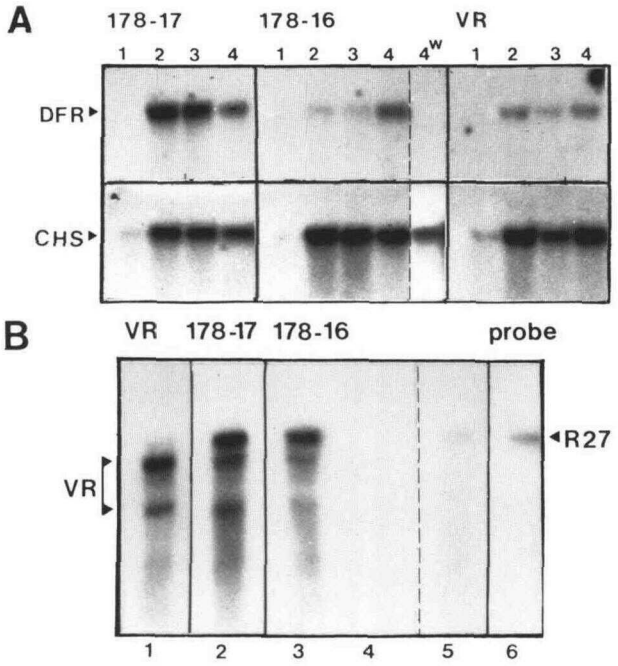

Figure 4. DFR and CHS Floral mRNA Steady-State Levels of VIP178 Transgenic Petunia.

(A) RNA gel blot analysis of floral RNA (5 μg, stage 1 to 4) from transformant 178-17 (pigmented), 178-16 (corolla pigmentation varies from almost wild type to white), and VR control plants was performed as described in Methods. DFR and CHS mRNA were detected using ^{32}P-labeled DFR or CHS cDNA, respectively. (For further details, see text.)

(B) RNase protection using 5 μg of floral RNA (stage 4) and the R27 DFR probe were performed as described in Methods. The probe was completely protected by VIP178 transcripts (indicated by R27), whereas protection with VR DFR RNA resulted in two subfragments (indicated by VR).

Lane 1, VR flowerbuds; lane 2, 178-17 flowerbuds; lane 3, 178-16 pigmented flowerbuds; lane 4, 178-16 unpigmented flowerbuds; lane 5, long exposure of lane 4 (10 days instead of 1 day); lane 6, DFR probe.

plants (van der Krol et al., 1988a). The gene construct consisted of a 0.8-kb CaMV 35S promoter fragment, a full-length CHS cDNA fragment, and the nopaline synthase 3′ tail fragment (Figure 2). Expression of this sense CHS gene in petunia did not result in a visible effect on flower pigmentation. We quantitated the pigment content of six independent CHS sense transformants in which the VIP103 copy number varied from one to three. These experiments did not show a significant increase in anthocyanin content in flowers of these sense transformants compared with untransformed control plants. This result suggests that the presence of an additional CHS gene does not lead to color enhancement. After reporting this result (van der Krol et al., 1988a), we were informed of similar experiments with reintroduced CHS genes that, unexpectedly, had given rise to pure white flowers as well as flowers with various patterns of white on a purple background with a frequency of 25% to 100% in independent experiments using different lines of petunia (see accompanying article: Napoli et al., 1990). This information, together with our earlier observation that high light intensities increase the degree of the antisense effect (van der Krol et al., 1989), prompted us to grow two sense CHS transformants supplemented with extra light. The result was new flowers with patches of white floral tissue (Figure 3A), confirming the results of Napoli et al. (1990). Analysis of extracts of the white parts by TLC showed the absence of anthocyanins and flavonols but the accumulation of coumaric acid, indicating that pigment synthesis is blocked at the CHS enzymatic step (data not shown).

The observed effect on flower pigmentation by constitutive DFR and CHS gene expression may be the consequence of a difference in timing of gene expression directed by the CaMV 35S promoter relative to that of endogenous DFR or CHS gene(s). Therefore, we tested the effect of a CHS gene that should have a timing of expression similar to that of endogenous CHS gene(s) during flower development. DNA fragments containing the CHS-A genomic clone were re-introduced into petunia VR plants. The CHS-A genomic clones VIP106 and VIP76 derive from clone VIP17 described by Koes et al. (1987) (Figure 2). VIP76 contains a 0.8-kb 5′ promoter region, 2.5 kb of CHS exon and intron sequences, and 3.9 kb of 3′-flanking DNA. VIP106 contains a 4.5-kb 5′ promoter region, 2.5 kb of CHS exon and intron sequences, and 0.5 kb of 3′-flanking DNA. Both genomic CHS clones were reintroduced into petunia VR plants using standard procedures (see Methods). Of the 20 plants regenerated that contain VIP106, one plant had an evenly reduced pigmentation of the corolla (Figure 3A; 106-16). One of the 15 plants containing the VIP76 construct had white flowers with small pigmented sectors (Figure 3A; 76-1A). TLC

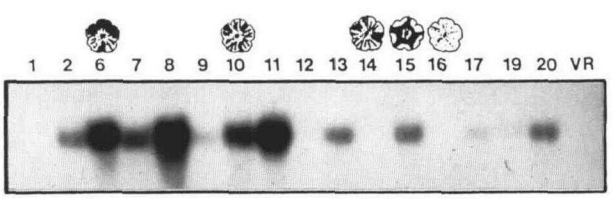

Figure 5. DFR mRNA Steady-State Level in Leaf Tissue of VIP178 Transgenic Petunia.

RNA analysis of leaf RNA (10 μg) was performed as described in Methods. DFR mRNA was detected using ^{32}P-labeled DFR DNA. The number of each VIP178 plant is indicated; VR represents leaf RNA from an untransformed petunia VR plant. The flower phenotype of transformants with altered floral pigmentation is indicated.

analysis of white floral tissue of this transformant showed the accumulation of coumaric acid, whereas incubation of this white floral tissue with naringenin-chalcone (the product of the CHS reaction) resulted in anthocyanin synthesis (data not shown). This result indicates that pigment synthesis is blocked at the CHS enzymatic step, whereas other enzymes involved in flavonoid biosynthesis are still present and active in these flowers.

Endogenous and Transgene CHS Expression in Transgenic Petunia

We analyzed CHS transcripts in transformants containing the CHS genomic clone VIP76. Figure 6B shows an overall reduction in CHS mRNA steady-state level compared with the VR control in transformant 76-1A, in which flower pigmentation is inhibited. The same RNA preparation, when tested for CHI or DFR transcripts, showed equal steady-state levels in the VR control plant and transformant 76-1A, indicating that the observed effect is specific for CHS gene expression. Transcripts from VIP103, VIP106, and VIP76 all contain CHS-A sequences that derive from the petunia strain V30. They can be distinguished from endogenous petunia VR CHS-A transcripts by primer extension experiments. Extension of a primer (EL-4) hybridizing to the first exon of CHS-A results in one major fragment (176 nucleotides, Figure 6A, lane 1) for V30 CHS-A mRNA and two major and one minor fragment for the VR CHS-A mRNAs (186 and 189 nucleotides and 181 nucleotides, respectively, Figure 6A, lane 2; in the VR hybrid, a duplication of the CHS-A gene has occurred after which the genes have slightly diverged; Koes et al, 1989b). Primer extension experiments on floral RNA from 76-1A and 76-2A show that the latter contains the V30 CHS-A transcript in addition to the endogenous VR transcripts (Figure 6A, lane 3). In 76-1A, both the endogenous VR as well as the introduced V30 CHS gene transcripts are absent (Figure 6A, lane 4). Longer exposure (Figure 6A, lane 5) reveals that both transcripts are present, but at severely reduced levels.

We determined the copy number of the CHS transgene in transformant 76-1A by DNA gel blot analysis. Five inserts of VIP76 were present in the genome of 76-1A, whereas transformant 106-16 contained approximately 10 copies of the VIP106 gene. Rehybridization of the same blot with a CHS-A gene probe showed that the endogenous CHS genes are unaltered in the 76-1A. This makes it unlikely that endogenous CHS genes are changed by insertion of VIP76 (data not shown).

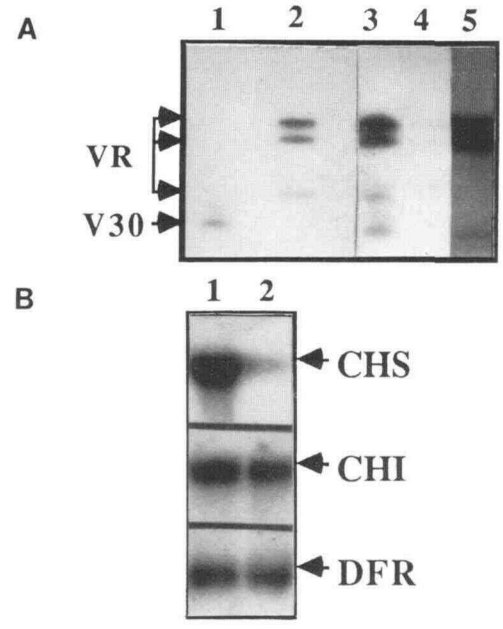

Figure 6. Expression of the Sense CHS Genes in Floral Tissue of Transgenic Petunia.

(A) Primer extension products analyzed on a 6% sequence gel. In the experiments, we used oligomer EL-4, which hybridizes to the 3′ end of the first exon of CHS mRNA and 5 μg of floral RNA (stage 4) from petunia V30 (lane 1), VR (lane 2), and VR transformants 76-2A (normal pigmented; lane 3) and 1A (reduced pigmentation; lanes 4, 5; see Methods).

(B) RNA gel blot analyses of floral RNA (5 μg, stage 4) from 76-1A (lane 2) and VR control (lane 1) plants were performed as described in Methods. The blot was hybridized with either ^{32}P-labeled CHS, CHI, or DFR DNA.

DISCUSSION

In this paper, we present the effect of introducing additional DFR or CHS gene copies into the genome of *Petunia hybrida*. A gene-specific block in pigment synthesis was observed in some of the transformants with a frequency that varied from 5% to 25% (our data) and higher (see accompanying article: Napoli et al., 1990). Because light conditions influence the final floral phenotype (van der Krol et al., 1989), there could be a seasonal effect on this frequency under greenhouse conditions. Some affected plants had a variable degree of pigmentation of flowers on one plant, but we also observed stable ectopic expression patterns in flowers of both the CHS and DFR sense transformants. These effects have also been observed after introducing antisense CHS genes into petunia plants (van der Krol et al., 1988a, 1989) and are ascribed to a position effect that influences spatial expression of the gene and its sensitivity to physiological parameters during flower development. Primer extension and RNase protection analyses of floral RNAs of the CHS and DFR sense transformants (Figures 4 and 6) revealed that overexpression of either CHS and DFR genes was tolerated without

affecting the flower phenotype. In addition, it was shown that, in transformants whose flowers exhibit ectopic color patterns, both the endogenous and the transgene-derived mRNA levels were drastically reduced in unpigmented floral parts. Although there is no direct evidence that the suppression of gene expression observed in the sense transformants was triggered by initial expression of the transgene, we think that this is indeed the case. This idea is supported by the data on the DFR transformant 178-16 (Figure 3B) in which DFR gene expression seems close to the point of collapse. Sometimes pigmented flowers had high transgene-derived DFR mRNA levels and sometimes unpigmented flowers had severely reduced DFR mRNA levels.

At this stage, the actual mechanism of sense inhibition of gene expression is unknown. Because of the high incidence of the phenomenon (this work and Napoli et al., 1990), trivial explanations involving somaclonal variation or homologous recombination can be ruled out. Although the effects of CHS and DFR genes in many ways resemble those of antisense CHS genes (van der Krol et al., 1990), rearrangements within the sense constructs were not detected and RNase protection experiments with riboprobes did not reveal the presence of such antisense molecules. The mechanism of suppression by sense genes may involve interference of RNA strands with the transcription process itself. The transcription process may be blocked by interaction of RNA with duplex DNA, resulting in a triple helix structure (Hogan and Cooney, 1989). Alternatively, interaction may occur with the DNA strands when they are separated during the transcription process. DNA modification processes, such as a cytosine methylation, could subsequently silence the gene permanently. The sense effect reported here may be related to a case of epistasis reported by Matzke et al. (1989), in which one copy of T-DNA suppressed the expression of an unlinked second copy present in the same cell. The specific suppression was caused by methylation. In the phenotypic mutants described in this paper, a double epistatic effect should have occurred, however. Experiments are in progress to test the alternatives. Recently, tobacco plants have been transformed with a constitutively expressed bean phenylalanine ammonia-lyase (PAL) gene, resulting in a dramatic reduction in PAL activity in some of the transgenic plants (C. Lamb, personal communication). Although no RNA data are currently available, the results suggest that a similar mechanism may operate in this case.

Finally, the question should be addressed whether the occurrence of the sense effect is limited to transgenic systems or might be a naturally occurring phenomenon. We note that the pigmentation patterns in petunia variety Red Star (Harder, 1938; Mol et al., 1983) resemble those of the antisense and sense transformants. Flowers on this mutant show pigmented and unpigmented sectors. Recent analysis of these Red Star mutants using chimeric CHS transgenes points to a sense effect occurring in the floral white sectors of these plants (I.M. van der Meer and A.R. Stuitje, unpublished data).

METHODS

Construction of VIP103, VIP76, VIP106, and VIP178

The construction of VIP103 has been described previously (van der Krol et al., 1988a). VIP76 and VIP106 derive from the petunia V30 CHS-A genomic clone VIP17, which has been described by Koes et al., (1989b). VIP76 was constructed by cloning a 7.2-kb EcoRI-SalI fragment from VIP17, containing the CHS-A gene with a 0.8-kb 5′-flanking region, into the EcoRI-SalI site of the binary vector BIN19 (Bevan, 1984). VIP106 was constructed by cloning an 8.0-kb XbaI fragment from VIP17, containing the CHS-A gene with a 4.5-kb 5′-flanking region, into the XbaI site of BIN19 (see also Figure 2).

VIP178 was constructed using a 1.3-kb EcoRI DFR cDNA fragment (from petunia R27; Beld et al., 1989). The EcoRI sticky ends were filled in using the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates. This blunt-ended fragment was cloned into the HincII site of M13mp7 so it could subsequently be isolated as BamHI fragment. This was cloned into the BamHI site of VIP103 from which the CHS sequences had been removed. The clone, which contains the BamHI DFR fragment in sense orientation to the CaMV 35S promoter, was called VIP178 (see also Figure 2).

Transformation and Growth of Plants

For transformation of petunia VR plants, the binary vectors with the different gene constructs were mobilized into *Agrobacterium tumefaciens* strain LBA 4404 using standard triparental mating techniques (Ditta et al., 1980). Transformants were obtained through the standard leaf-disc transformation method (Horsch et al., 1985). Transformed *Petunia hybrida* VR plants were grown under standard greenhouse conditions unless stated otherwise.

Isolation of Floral RNA and RNA Gel Blot Analysis

RNA was extracted from flowerbuds (stage 1 to 6 as defined by Koes et al., 1989a). To average out small differences in developmental stage of the flowerbuds, per stage, five flowerbuds taken from one plant were pooled. Nucleic acid isolation was done as described by Koes et al. (1987). RNA gel blots were made according to van Tunen et al. (1988). The filters (Highbond-N, Amersham Corp.) were hybridized according to the supplier's instructions with ^{32}P-labeled CHS, CHI, or DFR antisense RNA (see below).

RNase Protection and Primer Extension

RNase protection experiments were performed according to van Tunen et al. (1988). ^{32}P-labeled single-stranded CHS antisense RNA was synthesized in vitro using T7-polymerase (Promega Biotec), ^{32}P-UTP (Amersham), and the vector pTZ19U (Gene-

Scribe-Z TM, US Biochemical Corp.) in which an EcoRI-HindIII full-length CHS gene A cDNA was cloned (Koes et al., 1989b). The CHS probe is completely protected by the transcripts from VIP103, VIP76, and VIP106, whereas RNase protection with RNA from petunia VR results in subfragments due to small sequence divergence between CHS from petunia V30 and VR.

Similarly, ^{32}P-labeled DFR antisense RNA was synthesized from the vector pTZ18U in which the full-length DFR cDNA EcoRI fragment was cloned. This probe is completely protected by the transcripts of VIP178, whereas RNase protection with petunia VR DFR RNA results in subfragments due to small sequence divergence between DFR from petunia R27 and VR.

Primer extension experiments using oligomer EL-4 (5′-dGATCAACACAGTTTGTAGG-3′) and 5 μg of floral RNA (stage 4) were performed as described by Koes et al. (1989b). The lengths of the extension products were determined by performing primer extensions with EL-4 on a single-stranded CHS-A template in the presence of the four dideoxynucleoside triphosphates. In petunia V30, the experiments revealed the presence of one major fragment of 176 nucleotides, whereas, for the petunia VR hybrid, two major and one minor fragment were found of 186, 189, and 181 nucleotides, respectively.

DNA Gel Blotting

DNA gel blots of genomic plant DNA were made according to Koes et al. (1987). DNA was isolated from leaf tissue and 10 μg was digested with restriction endonucleases and electrophoretically separated on 0.8% agarose gels. DNA was transferred onto Highbond-N membranes as recommended by the supplier (Amersham). The blot was hybridized according to the supplier's protocol with DNA labeled through nick translation (Maniatis et al., 1982).

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